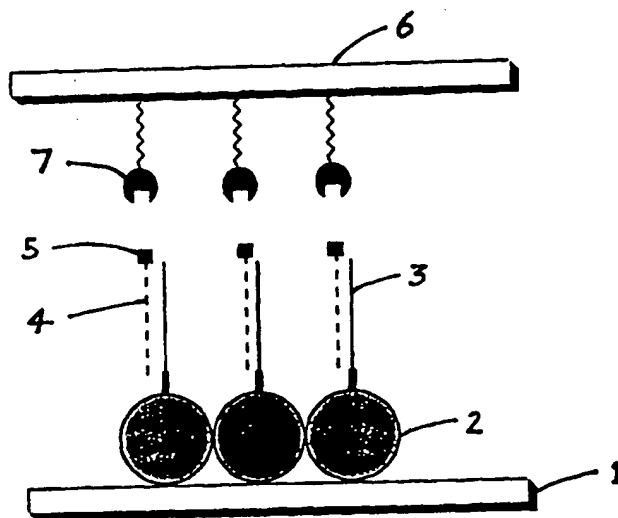




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<p>(21) International Application Number: PCT/GB99/03691</p> <p>(22) International Filing Date: 8 November 1999 (08.11.99)</p> <p>(30) Priority Data:</p> <table border="0"> <tr> <td>9824441.1</td> <td>6 November 1998 (06.11.98)</td> <td>GB</td> </tr> <tr> <td>9827581.1</td> <td>15 December 1998 (15.12.98)</td> <td>GB</td> </tr> <tr> <td>9919605.7</td> <td>18 August 1999 (18.08.99)</td> <td>GB</td> </tr> </table> <p>(71) Applicant (for all designated States except US): SOLEXA LTD. [GB/GB]; 38 Jermyn Street, London SW1Y 6DN (GB).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): BALASUBRAMANIAN, Shankar [GB/GB]; University of Cambridge, Dept. of Chemistry, Lensfield Road, Cambridge CB2 1EW (GB). KLENERMAN, David [GB/GB]; University of Cambridge, Dept. of Chemistry, Lensfield Road, Cambridge CB2 1EW (GB).</p> <p>(74) Agent: GILL JENNINGS & EVERY; Broadgate House, 7 Eldon Street, London EC2M 7LH (GB).</p>		9824441.1	6 November 1998 (06.11.98)	GB	9827581.1	15 December 1998 (15.12.98)	GB	9919605.7	18 August 1999 (18.08.99)	GB	<p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report.</p>
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(54) Title: A METHOD FOR REPRODUCING MOLECULAR ARRAYS



(57) Abstract

A method for producing an array of molecules immobilised on a substrate, comprises the steps of: forming a hybrid array from an array of first molecules (3) immobilised on a first substrate (1) and second molecules (4), i.e. the molecules to be immobilised, thereby defining a spatial array of the second molecules; bringing into close proximity the first substrate (1) and a second substrate (6), wherein the second substrate (6) and the second molecules (4) can be mutually linked; by linking them, causing the second molecules (4) to be printed onto the second substrate (6) while retaining the spatial array; and separating the respective substrates.

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A METHOD FOR REPRODUCING MOLECULAR ARRAYS

Field of the Invention

This invention relates to a method for reproducing, or cloning, molecular arrays.

Background of the Invention

5 Much of cellular and molecular biology is based on specific non-covalent interactions between molecules, sometimes referred to as molecular recognition. These interactions are not permanent and are based mainly on hydrophobic interactions and hydrogen bonds, so that the binding together of two molecules is reversible. A molecule that recognises specifically another molecule can be defined as a cognate molecule or
10 complement molecule. For a strand of DNA, this cognate can be a strand of complementary sequence. The molecule cognate can also be a protein, e.g. a transcription-regulating protein which binds a sequence of DNA or a zinc finger. Alternatively, the cognate molecule may be an antibody which recognises an antigen, an enzyme binding a particular substrate or a receptor binding a ligand, or *vice versa*.

15 Molecular recognition has been exploited in many laboratory techniques. For example, in western blotting, separated biomolecules may be transferred from one substrate to another for subsequent interrogation with a cognate. However, while this transfer may retain the spatial organisation from the first substrate, it does not allow repeated transfer of, say, a close-packed addressable array of biomolecules.

20 WO-A-93/17126 discloses a binary oligonucleotide array, and the transfer of hybridised oligonucleotides, by a blotting technique. The transfer is non-specific. A non-specific transfer of colony material, using a colony lift membrane, is disclosed in US-A-5491068.

25 WO-A-95/12808 discloses the selective transfer of DNA from solution to different binding locations, by applying a relatively positive potential, and then a relatively negative potential, at one location. The first step of the process binds target and non-target DNA; the second releases non-target DNA.

30 US-A-5795714 discloses a method for transferring, to a second surface, a DNA molecule complementary to an arrayed DNA molecule. Transfer is carried out by bringing the second surface into direct contact with a solution comprising the complementary DNA, the solution being contained within discrete vials present on a first

substrate. The preferred embodiment requires the use of avidin/biotin interactions to aid transfer. The method relies on diffusion to transfer the complementary DNA. Any lateral diffusion will limit the resolution that may be achieved when carrying out transfer from high density arrays.

5 Summary of the Invention

According to the present invention a method for producing an array of molecules immobilised on a substrate, comprises the steps of:

forming a hybrid array from an array of first molecules immobilised on a first substrate and second molecules, i.e. the molecules to be immobilised, thereby defining
10 a spatial array of the second molecules;

bringing into close proximity the first substrate and a second substrate, wherein the second substrate and the second molecules can be mutually linked;

by linking them, causing the second molecules to be printed onto the second substrate while retaining the spatial array; and

15 separating the respective substrates.

In one preferred embodiment of the invention, molecules of a particular class can be attached to a surface using a strong linkage such as a covalent bond to form a spatially addressable array. If this surface is then exposed to a variety of different cognates and allowed to reach near equilibrium, then a cognate will bind to the molecule on the surface
20 for which it has the strongest affinity. Unbound molecules can then be removed by washing. Since the cognate is only attached to the molecule it recognises by non-covalent interactions, it can be transferred to a second surface in close proximity by applying a suitable electric field (under conditions where it has a net positive or negative charge).

25 In another preferred embodiment of the invention, the cognates include a covalent or non-covalent coupling group at one terminus. After contacting the molecules on the surface, the cognates can be transferred to a second surface by contact printing. The second surface should be first treated to include complementary covalent or non-covalent coupling groups to react with those on the cognate terminus. No electric charge is
30 required to effect transfer.

Thus, if the first surface has particular regions each containing different molecules, the cognate molecules can be printed onto the second surface to produce a spatial array of cognates. It is preferable, but not essential, for the cognates to bind to the second surface with high affinity; this is only essential if it is desired to repeat the process on the arrayed cognates, to form a molecular positive array, e.g. to form a copy of the original array. For example, a stronger bond may be formed by forming a covalent bond between the cognates and the second surface or through a strong non-covalent interaction, e.g. an avidin-biotin bond.

The novel method is applicable to both single molecule and many molecule arrays, i.e. arrays of distinct individual molecules and arrays of distinct regions each comprising multiple copies of one individual molecule. The advantages of this method are numerous. In particular, it means that only one spatially addressable array needs to be made, and then multiple copies can be made for screening and diagnostics.

The molecule array may be characterised prior to printing. For example, the array may be spatially addressed by, say, sequencing, so that each molecule on the array is known.

Description of the Invention

The first or master molecule array used in this invention may comprise proteins immobilised on a solid surface, e.g. antibodies or enzymes. The proteins are capable of interacting with other molecular species (cognates), e.g. proteins, small molecules or polynucleotides which may then be transferred to a second substrate. For example, the arrayed proteins may be zinc finger proteins which are capable of binding polynucleotides with sequence selectivity; see Choo *et al*, PNAS USA, 91:11163 (1994). In certain circumstances, the cognates may not be known, and further characterisation may be required to determine precisely what activity or function the cognate performs.

Alternatively, the arrayed molecules may be polynucleotides. The term "polynucleotide" is used herein to refer to DNA, RNA and synthetic derivatives or mimics capable of interacting with DNA and RNA, e.g. thioates, amidates and PNAs. Whatever the molecules in the first array, the term "cognate" is used herein to refer to a molecule that has specific recognition for a molecule different in structure to itself. The respective molecules will typically have complementary portions.

In particular, this invention allows multiple copies of a molecule (e.g. DNA or RNA) array to be produced from a single molecule array (e.g. polynucleotide array) (master copy) which may be spatially addressed. This method is based on making the complementary (say) DNA to DNA on the array, e.g. using DNA polymerase or by direct
5 hybridisation from a mixture of oligonucleotides, so that the complementary DNA is hybridised to the original DNA in the array, and then printing the complementary DNA onto a second surface. In this step, a second substrate is brought into proximity with the hybrid array; then, e.g. by charging the second substrate, the complementary polynucleotides are printed on it.

10 In a separate embodiment of the invention, it is possible that the transferred molecule is not the cognate *per se*, but, rather, is a product of a reaction between the cognate and arrayed molecule. For example, the arrayed molecule may be an enzyme which reacts with its substrate (cognate) to form a product. As this is a localised effect, it will be possible to transfer the product onto the second surface. In this case, the
15 product may be understood as the molecule to be transferred.

In a related embodiment, the arrayed molecule may "capture" its cognate but the cognate undergoes reaction with a further substrate, and the product of this reaction is transferred to the second surface. For example, the arrayed molecule may capture a specific enzyme in a manner that retains the active site. The product of the enzyme-
20 catalysed reaction is then transferred to the second substrate.

In a further embodiment, the first and second molecules may not hybridise directly. Indirect associations may comprise phage-bacterium-phage or antibody-cell-antibody interactions (examples of the invention in which the respective arrayed molecules may be the same, or different).

25 By way of illustration only, the invention will be now described with reference to DNA polynucleotides, and to spatially addressable arrays. The production and uses of such arrays are described in PCT/GB99/02487, the contents of which are incorporated herein by reference. The density of such arrays may be at least 10^4 , e.g. at least 10^5 or 10^6 entities/cm², up to 10^9 entities/cm² or more, and comprising the same or different
30 molecules optionally immobilised on beads (which can typically be c. 1 μ m beads, packed

at a density of 10^8 per cm^2). The fact that the molecules can be different gives broad applicability to the invention.

In one embodiment of the invention, a DNA array, e.g. on glass or silicon, is copied by hybridisation of a library of single-stranded DNA, under conditions such that
5 members of the library hybridise to their complement strands of DNA on the array. Alternatively, an array complementary to the master copy is made by enzymatic synthesis using a DNA polymerase and a suitable primer and dNTP's.

Once the DNA array has been made, any non-hybridised DNA may be removed by washing. This results in all or most of the DNA in the spatially addressable array
10 being hybridised to its complementary DNA. The complementary array can now be transferred and attached to the second substrate.

The respective means of attachment of DNA to the first and second substrates should preferably be orthogonal, in order to achieve clean transfer. If the same means was used, transfer might not occur in the event that the complementary DNA can bind
15 to the master. Assuming that this can be prevented, the respective attachments can be of similar or different strengths provided that each is greater than the hybridisation strength of the DNA-DNA duplex. The latter can of course be reduced by destabilising it, by known procedures such as heating, or by changing salt concentration.

The DNA in the original array is preferably attached to the first substrate surface
20 by a strong bond such as a covalent bond or via avidin-biotin which has similar strength to a covalent bond. In order to achieve bonding to the second substrate, the complementary DNA preferably has a terminal group that is chemically-reactive, or activatable, so that it reacts with, and thus becomes attached to, the second substrate surface. When the DNA hybrid is formed, this terminal group may be positioned so that
25 it is furthest away from the surface of the first substrate. For example, the terminal group may be biotin or avidin, in which case the second substrate surface is covered in a layer of avidin or biotin, respectively, for attachment. An example of an activatable group is "caged" biotin, and this can be photoactivated, during the transfer process, to achieve printing on the second substrate. When the respective substrates are separated, the
30 relatively weak bond between the hybridised molecules is broken and the respective molecules are held by the respective substrates. Spatial resolution is maintained.

As indicated above, transfer may be achieved without contacting the second substrate with the cognates in the hybrid array, but under an electric field. In this case, both surfaces should be conducting. The first surface may be a metal or doped semiconductor such as silicon. The master array may be attached to the surface by a covalent linkage (preferred) or a strong specific interaction. Since the specific interaction with the cognate molecules often includes a number of hydrogen bonds, the transfer may be performed in an electrolyte; for example, a DNA duplex is stable in salt solution but unstable in pure water.

On application of a sufficiently strong field, the cognates will transfer from the hybrid array to the other and can then be anchored to the copy surface *via* a specific interaction. In addition, ions will move towards the electrodes, positive ions to the negative electrode and *vice versa*. This electrolysis may damage the master array if it proceeds for too long. In order to keep the potential required low and to ensure good spatial transfer, the electrodes can be spaced apart by non-conducting spacers such as Teflon, e.g. by 0.1-10 μm , often 5-10 μm , i.e. as close as possible without shorting of the electrodes. A potential of 1 mV to 1 V may be applied to the electrodes for a short period, e.g. 1 ms to 1 s, for transfer of the cognates from the master to the copy, without causing any damage of the master. The potential and time will depend on the spacing between the electrodes and the electrolyte ionic strength and can be optimised. The polarity of the potential applied will depend on the charge on the cognates. The cognates can be attached to the copy electrode by either non-specific interactions or by having a suitable layer of molecules on the electrode and a suitable group on the cognates, so that a specific bond can be formed once the transfer has occurred.

The second substrate is preferably a semiconductor, e.g. silicon or a gold-coated surface. The transfer may be done in the presence of a material that mediates the transfer of the complementary polynucleotides, e.g. a polymer gel or a thin film of, say, water or some other suitable liquid (although transfer in air or in a vacuum may also be possible). Solution conditions or heating of the array during the printing process may help ensure good transfer.

Printing on the second substrate may be facilitated by any suitable means. For example, the second substrate is or can be charged. Charging may be by static electricity.

Preferably, a positive potential is applied to the semiconductor surface, by means of a suitable source of voltage. The effect of this is to attract the negatively-charged phosphate backbone of the hybridised DNA and pull it onto the semiconductor surface. As has already been demonstrated, hybridised DNA can be removed at a modest potential, of 300 V.m⁻¹; see PNAS USA 94:119 (1994).

It is very desirable that the surfaces of the respective substrates are close together, in order to obtain exact copying and for there to be no problem of adjacent elements in the array switching position during the printing process. Closeness also makes it much easier to produce a sufficiently strong electric field, to attract the hybridised DNA onto the surface of the second substrate.

Any semiconductor or insulator surface that can be sufficiently polarised to attract the hybridised DNA is suitable. The second substrate may also be, say, a thin layer of glass such as a coverslip, used with a metal or other electrode directly behind it, in order to apply the positive potential. The second substrate is preferably not a metal, since that may quench any fluorescence when the array is used with fluorescent probes.

In an alternative aspect of the invention, the transfer may be effected merely by bringing the two surfaces together, so that the second surface can bond directly to the molecules to be transferred. No applied field is necessary. In this method it is desirable for the cognates to have a coupling group attached with a complementary coupling group attached to the second surface. Therefore, transfer is mediated by the interaction between the respective coupling groups, providing deformed points of attachment, and spatial integrity is maintained. Suitable coupling groups are as defined above, i.e. biotin/avidin, thiol linkers, etc.

The surfaces of both substrates should be as flat as possible. Suitable silicon wafers are readily available.

Either or each substrate may comprise beads to which the DNA is attached. In this case, the beads may be used to keep the two surfaces apart; one surface may be placed directly on top of the other, their separation being defined by the diameter of the beads.

Beads are particularly preferable when the process uses contact printing without any electric charge. The presence of the beads between the two surfaces will facilitate

transfer since the contact will be between the top of the bead and the surface onto which printing is taking place. It may also be beneficial to have a non-rigid surface, for example by reducing the thickness of the substrate or by using a material that is deformable e.g. thin plastics.

5 Following transfer, the original, master copy of DNA, which is attached by a stronger bond to the surface of the first substrate than to the complementary DNA, remains attached. The complementary DNA is printed onto the second substrate which is then removed, leaving the original master array intact, ready for further printing. If necessary or desired, this process may be repeated on the complementary DNA copy, to
10 obtain an exact copy of the original array.

 In this context, reference may be made to Fig. 1. This shows a first substrate 1 carrying beads 2 on which there is an array of DNA molecules 3. The DNA molecules 3 are covalently attached to the beads. Complementary DNA molecules 4 have a reactive
15 functionality 5. A second substrate 6 is modified to carry groups 7 that react with the complementary DNA molecules, to bind them covalently.

 It will be apparent to the skilled person that the methods of the present invention can be applied to any molecular species involved in molecular recognition. For example, antibody-antigen recognition may be adapted in the invention, DNA binding proteins may also be used, either as the immobilised template array, or as the cognate molecules, and
20 enzymes and their substrates may also be used.

 It will also be apparent that a principal advantage of this method is that only one master array needs to be made and then multiple copies can then be printed. This will greatly increase the speed of production of arrays, and enable them to be widely used for diagnostics, genotyping and expression monitoring.

25 The following Example illustrates the invention.

Example

Preparation of Glass Slides

 Glass slides to which DNA was to be transferred were cleaned by immersion in 1:1 conc. HCl:MeOH for 1 h, rinsed in Milli-Q water, immersed in conc. H₂SO₄ for 1 h
30 and rinsed in water again. Cleaned slides were stored in mQ water.

The slides were silanised with amino-functionalised silane reagent, N-[3-(trimethoxysilyl)propyl]ethylenediamine (DETA). Silanisation of cleaned glass substrates was performed using a 1% solution of DETA in 1 mM glacial acetic acid for 1 h. The slides were rinsed with mQ water, dried with N₂ and baked at 120°C for 5 minutes.

The silanised slides were then reacted with SMCC, a heterobifunctional linker capable of reacting with amine and thiol groups (see Fig. 2). 15 mg (45 µmoles) SMCC was dissolved in 200 µl DMSO. This was diluted to 120 ml in 80:20 MeOH:DMSO. Silanised slides were immersed in the solution for 3 h at RT, then rinsed well with mQ water and dried under N₂. The maleimide-derivatised slides were stored in a vacuum desiccator.

Control Experiment

A slide was tested to ensure that the maleimide surface was reactive towards thiols. A 5'-SH, 3'-TMR 20-mer oligonucleotide (SEQ ID No. 1) was used. DTT was removed from the sample (as it will interfere with the reaction) by passing the sample down a NAP-5 gel filtration column. 500 µl of the thiol oligo solution was placed on an SMCC-reacted glass slide, and 500 µl on a control glass slide, and placed in a humid environment for 2 h at RT. The slides were then rinsed in mQ-water and placed in SPSC buffer (50 mM NaP_i, 1 M NaCl) for 12 h, in order to remove any DNA that was not covalently attached to the surface. The slides were rinsed, dried under N₂, and visualised with a FluorImager (488 nm excitation, 570 nm filter).

Transfer

An ethanol solution of 1.0 µm silica beads, to which was attached a 20-mer DNA sequence (SEQ ID No. 2), was spotted onto a cleaned glass slide, and the EtOH allowed to evaporate to form a monolayer of beads. 5 such slides were prepared. 25 µl of a 10 µM solution of the 5'-SH, 3'-TMR in 10 mM KPi, 100 mM NaCl, 1 mM DTT was added to the circular patch of beads on each slide and allowed to hybridise for 1 h at RT. The slide was rinsed well (5 x 5 ml washes) with buffer (10 mM KPi, 100 mM NaCl) in order to remove DTT and unhybridised oligo. The reactive SMCC-derivatised slides were then carefully placed over the slide having the hybridised duplex on the beads. 0, 1, 2, 3 and 4 extra glass slides were placed on top of the respective SMCC slides, in order to

increase the weight, and therefore the amount of contact, with the beads. The slides were placed in a humid environment for 2 h at RT, after which the reactive top slide was carefully removed and placed in SPSC buffer for 12 h to remove any oligo not covalently attached to the surface. The slides were rinsed, dried and visualised using the
5 FluorImager (at 488 nm excitation).

Results

DNA transfer from the beaded array to the glass surface has been achieved. The circular images observed correspond to the shape of the original patch of beads on the surface. Based on the control experiment, the observed fluorescence can only represent
10 fluorescent oligonucleotide that has been transferred from the beaded array and covalently attached to the opposite surface.

CLAIMS

1. A method for producing an array of molecules immobilised on a substrate, which comprises the steps of:
forming a hybrid array from an array of first molecules immobilised on a first
5 substrate and second molecules, i.e. the molecules to be immobilised, thereby defining a spatial array of the second molecules;
bringing into close proximity the first substrate and a second substrate, wherein the second substrate and the second molecules can be mutually linked;
by linking them, causing the second molecules to be printed onto the second
10 substrate while retaining the spatial array; and
separating the respective substrates.
2. A method according to claim 1, wherein, in the hybrid array, the first and second molecules are indirectly associated.
3. A method according to claim 1, wherein the first and second molecules include
15 complementary portions.
4. A method according to any preceding claim, wherein the first molecules are proteins.
5. A method according to any preceding claim, wherein the second molecules are proteins, e.g. zinc finger proteins.
- 20 6. A method according to claim 3, wherein the first and second molecules are polynucleotides.
7. A method according to claim 6, wherein the second molecules comprise a library of single-stranded polynucleotides.
8. A method according to claim 6, wherein the second molecules in the hybrid array
25 are formed *in situ*, using a polymerase and the nucleotide triphosphates.
9. A method according to any preceding claim, wherein the linking comprises covalent or avidin-biotin binding.
10. A method according to any preceding claim, wherein the first molecules are immobilised on microscopic beads bound to a solid support.

11. A method according to any preceding claim, wherein the second substrate and the second molecules are brought into contact and linked, and the respective substrates are then separated.
12. A method according to any of claims claim 1 to 10, wherein the second molecules
5 are printed onto the second substrate under the application of an electric field.
13. A method according to claim 12, which additionally comprises introducing, between the first and second substrates, a material that mediates the transfer of the second molecules from the hybrid array to the second substrate.
14. A method according to claim 12 or claim 13, wherein one or each substrate
10 comprises a semiconductor surface.
15. A method according to claim 14, wherein the semiconductor surface comprises silicon.
16. A method according to any preceding claim, wherein the facing surfaces of the respective substrates are flat.
- 15 17. A method according to any preceding claim, wherein either or each array comprises a plurality of different molecules, at a density of at least 10^5 per cm^2 .
18. A method for producing a copy of an array of first molecules immobilised on a first substrate, which comprises the steps of any preceding claim, and repeating those steps using the first molecules to form the hybrid array and a third substrate on which to
20 print the first molecules.
19. A method according to any preceding claim, which is repeated any desired number of times, thereby producing a plurality of copies of immobilised molecules having the spatial array.

1/1

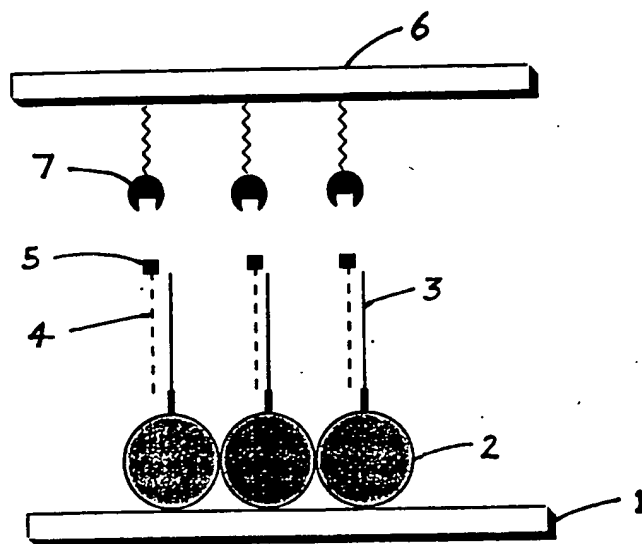


FIG. 1

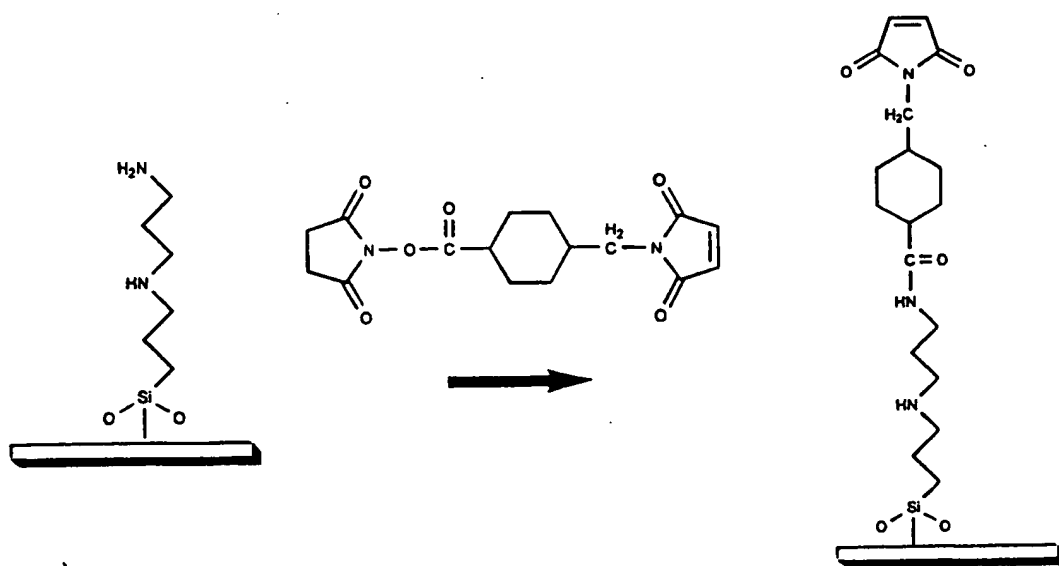


FIG. 2

SEQUENCE LISTING

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<120> A METHOD FOR REPRODUCING MOLECULAR ARRAYS

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<210> 2

<211> 20

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence:
Oligonucleotide

<400> 2

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20

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 99/03691

A. CLASSIFICATION OF SUBJECT MATTER IPC 7 B01J19/00 C12Q1/68		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 7 B01J C12Q		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 93 17126 A (THE PUBLIC HEALTH RESEARCH INSTITUTE OF THE CITY OF NEW YORK, INC.) 2 September 1993 (1993-09-02) abstract page 2, paragraph 3 -page 4, paragraph 1 page 14 page 16, last paragraph -page 21, paragraph 1 figures 6,7A,7B	1,3,6-8, 11,16,17
A	---	2,18,19
A	US 5 491 068 A (THOMAS L. BENJAMIN ET AL.) 13 February 1996 (1996-02-13) abstract column 3, line 39 - line 52 column 4, line 10 - line 63 column 5, line 61 -column 7, line 8 --- <div style="text-align: right;">-/--</div>	1-19
<div style="display: flex; justify-content: space-between;"> <input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex. </div>		
<div style="display: flex;"> <div style="flex: 1;"> <p>* Special categories of cited documents :</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="flex: 1;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
Date of the actual completion of the international search <div style="text-align: center;">21 February 2000</div>		Date of mailing of the international search report <div style="text-align: center;">29/02/2000</div>
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer <div style="text-align: center;">Stevnsborg, N</div>

INTERNATIONAL SEARCH REPORT

International Application No.

PCT/GB 99/03691

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